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Effect of *Citrus bergamia* juice on human neuroblastoma cells *in vitro* and in metastatic xenograft models

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ABSTRACT

Neuroblastoma is the most common extracranial pediatric solid tumor with poor prognosis in children with disseminated stage of disease. A number of studies show that molecules largely distributed in commonly consumed fruits and vegetables may have anti-tumor activity. In this study we evaluate the effect of *Citrus bergamia* (bergamot) juice (BJ) *in vitro* and in a spontaneous metastatic neuroblastoma SCID mouse model. Qualitative and quantitative characterizations of BJ flavonoid fractions were performed by RP-HPLC/PDA/MS. We show that BJ significantly affects SK-N-SH and LAN-1 cell proliferation *in vitro*, but fails to reduce primary tumor weight *in vivo*. Moreover, BJ reduced cell adhesiveness and invasion of LAN-1 and SK-N-SH cells *in vitro* and the number of pulmonary metastases under consideration of the number of tumor cells in the blood in mice inoculated with LAN-1 cells *in vivo*. These effects without any apparent sign of systemic toxicity confirm the potential clinical interest of BJ and lay the basis for further investigation in cancer.

1. Introduction

Neuroblastoma (NB) is the most common tumor in children less than one year of age worldwide. Each year, approximately 1500 cases occur in Europe and 700 in the USA and Canada, accounting for about 28% of all cancers diagnosed in European and USA infants [1]. NB is a malignant embryonic tumor of the neural crest cells and therefore can develop anywhere along the strands of the sympathetic nervous system [2]. Hematogenous metastasis is present in about half of NB patients and is responsible for many NB deaths. Although aggressive and intensive multimodality therapies (surgery, cytotoxic chemotherapy, radio-metabolic treatment) have produced some improvements in the overall cure rate of NB patients, the prognosis of patients with metastatic NB remains poor. Thus, novel therapeutic strategies to ameliorate the prognosis of NB patients are required.

Plant kingdom has always been an attractive source of novel anticancer drugs resulting in the fact that about 50% of anticancer drugs are natural or semisynthetic products [3]. Epidemiologic studies provided evidence that *Citrus* consumption is associated with a reduced all-cancer incidence, although significant results were obtained only for prostate and pancreatic cancers [4]. In contrast, some studies did not find an association between *Citrus* fruit intake and cancer prevention [5,6]. However, several *in vivo* studies suggest that *Citrus* juices have a potential in cancer prevention. Thus, it has been documented that mandarin juice may reduce both chemically-induced rat colon carcinogenesis [7] and lung cancer formation [8]. Moreover, orange juice may decrease both chemically-induced mammary tumor burden and azoxymethane-induced colon cancer in rats [9,10]. Further, grapefruit juice shows a suppressive effect on *in vivo* induced colon carcinogenesis [11].

Citrus bergamia Risso et Poiteau (bergamot) is a small tree belonging to the family Rutaceae. 90% of bergamot worldwide

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production occurs in the southern coasts of Calabria region (Italy), where the microclimatic characteristics are particularly suitable for its cultivation. Bergamot fruit is used mostly for the extraction of its essential oil from the peel, a basic component of fragrances. Bergamot juice (BJ), which is obtained from the endocarp after essential oil extraction, is considered just as a secondary and discarded product with disposal costs. Over the past few years BJ attracted large attention as a result of its remarkable content of flavonoids, known for their beneficial effects.

Flavonoids are polyphenolic compounds present in vegetables, especially in the genus *Citrus*, characterized by a common benzo-γ-pyrone structure. More than 8000 compounds of flavonoid structure have been described. The large number of molecules arises from various combinations of multiple hydroxyl and methoxyl groups substituting the basic flavonoid skeleton. Numerous pre-clinical and epidemiological studies point to a possible protective effect of flavonoids against cardiovascular and malignant diseases, linked to their abilities to inhibit enzymes involved in cell activation [12].

Recently, we have shown that BJ reduces growth rates of different cancer cell lines, by mechanisms that in SH-SY5Y neuroblastoma cells are linked to an early impairment in cell adhesive and migratory processes [13]. However, until now the anticancer properties of BJ *in vivo* have not been investigated. On these bases, the present study was designed to evaluate the effect of BJ both *in vitro* and in an experimental model of spontaneous metastasis formation of human NB cells xenografted into immunodeficient mice.

2. Materials and methods

2.1. Plant material

Bergamot fruits were collected from *C. bergamia* cultivation located in Bovalino (Reggio Calabria, Italy). The fruits were hand-squeezed and small aliquots of the juice (BJ) were stored at −20 °C. BJ was defrosted and filtered (0.22 μm pore size) before use. For cell culture experiments, the pH of BJ was adjusted to 7.4 and subsequently diluted in culture media until the desired concentrations are achieved.

2.2. Quali-quantitative evaluation of flavonoids in bergamot juice

The juice was analyzed without any pre-treatment: the juice was centrifuged and then filtered on Acrodisc filter 0.22 μm. The sample was analyzed in triplicate by RP-HPLC. Since the pH of the BJ is roughly 3.5–3.8, all the mobile phases have been adjusted to the appropriate pH value (pH 3) with formic acid in order to suppress the ionization of the phenolic groups [14].

LC analyses were carried out using a Shimadzu Prominence LC-20A system (Shimadzu, Milan, Italy), including a CBM-20A controller, two LC-20 AD dual-plunger parallel-flow pumps, a DGU-20A3 on-line degasser, and a CTO-20A column oven. Data were acquired and processed by LCsolution Version 1.21 SP1 software (Shimadzu). An SPD-M20A UV detector and an LCMS-2020, through ESI interface (Shimadzu), were employed for quantification and characterization of bioactive molecules, respectively. MS data acquisition was performed by the LCMSsolution Ver. 3.30 software (Shimadzu).

LC analyses were carried out on an Ascentis Express C18, 150 × 4.6 mm I.D. with a particle size of 2.7 μm (Supelco, Bellefonte, PA). The injection volume was 2 μl: mobile phase consisted of water/formic acid (99.9:0.1, v/v; solvent A) and acetonitrile/formic acid (99.9:0.1, v/v; solvent B). The stepwise gradient profile was as follows: 0 min, 5% B, 40 min, 25% B, 60 min, 100% B, 70 min, 100% B, 73 min, 5%, and 80 min 5% B. Flow-rate was 0.7 ml/min. Data were acquired using a photodiode array detector in the range 190–400 nm and the chromatograms were extracted at 283 nm and 325 nm. Time constant was 0.64 s and sample frequency 1.5625 Hz. Data acquisition was performed by Shimadzu LCsolution software ver 3.3.

MS acquisition was performed using ESI in negative mode. ESI conditions: mass spectral range, m/z 100–700; interval, 0.5 s; scan speed, 938 amu/s; nebulizing gas (N2) flow, 1.5 l/min; ESI temperature, 350 °C; heat block, 300 °C; DL (desolvation line) temperature, 300 °C; DL, voltage, −34 V; probe voltage, +4.5 kV; Qarray voltage, 1.0 V and detection gain, 1.05 kV.

To quantify the flavonoid content in the BJ, calibration curves have been constructed by using each single standard. Five different concentrations of each component in the range between 100 and 0.2 mg/l (stock solution of 1000 mg/l in methanol) were analyzed for five consecutive times by HPLC under the same chromatographic conditions optimized for the sample. Limit of detection (LOD) and limit of quantification (LOQ) values were also calculated as reported [14].

2.3. Cell lines, proliferation assay and cytotoxicity study

SK-N-SH and LAN-1 human NB cell lines were cultured as described for other NB cell lines [15] at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 supplemented with 10% (vol/vol) heat inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. At confluence, cells were routinely harvested using 0.05% trypsin–0.02% EDTA and replaced in 75 cm^2 tissue culture flasks. All chemicals were from Gibco (Paisley, Scotland and Milan, Italy).

Cell proliferation was quantified by the MTT assay [16] with modifications. Cells were plated into 96-well plates at a density of 15 × 10^3 cells/ml (LAN-1) or 6 × 10^3 cells/ml (SK-N-SH) and cultured for 24 h before administration of growing percentage of BJ (from 1% to 10%). Each concentration was eightfold tested and at least four independent experiments were carried out. The control cells received only fresh medium. After incubation for 24, 48 and 72 h, the plates were centrifuged to collect the floating cells in the media, and the supernatants in each well were replaced with 100 μl fresh medium without phenol red containing 0.5 mg/ml of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich; Milan, Italy). The plates were returned in the incubator for 4 h and gently shaken occasionally. Then, the medium was removed and 100 μl of ethanol/dimethyl sulfoxide (DMSO) 1:1 lysis buffer was added to solubilize the formed formazan crystals (MTT metabolic product) that were spectrophotometrically quantified with a microplate spectrophotometer (Bio-Rad Laboratories, Milan, Italy). Absorbance of

untreated controls was set to 100% and absorbance of BJ-treated cells was expressed as a percentage relative to the control.

Cell growth was also detected by the cell count assay. Briefly, SK-N-SH and LAN-1 cells were seeded onto 6-well plates (1×10^5 cells/well for the SK-N-SH and 3×10^5 cells/well for the LAN-1 cells) and the next day treated with the BJ ranging from 1% to 10%. Then, cells were harvested, centrifuged and resuspended in a known amount of culture medium containing the trypan blue dye for distinguishing live cells from death. Aliquots of cell suspensions were put in a Neubauer hemocytometric chamber and cells were count by a common optical microscope.

Possible cytotoxic effects of BJ was assessed by a trypan blue dye (0.4% v/v) exclusion test and cell death was reported as the percentage of stained (non-viable) *vs* total cells counted [17].

All the tests were carried out in eightplicate (MTT) or in triplicate (cell count and trypan blue) and repeated three times.

2.4. Laminar flow assay

Laminar flow was produced in IBIDI microslides VI (IBIDI GmbH, München, Germany; ibiTreat-pretreatment; width: 3.8 mm, height: 0.4 mm, volume of each capillary: 30 μl) connected to a syringe pump (model 100 Series; KD Scientific, Holliston, MA, USA) and cell movement was observed with an inverted microscope (Zeiss, Axiovert 200). SK-N-SH and LAN-1 cells were treated with 5% BJ for 24 h and then detached, span down and resuspended in cell culture medium (1×10^5 cells/ml). Microslides were coated with recombinant human (rh) E- (5 μg/ml) or P-(50 μg/ml) selectin Fc-chimeras (R&D Systems GmbH, Wiesbaden, Germany) diluted in PBS [18]. A control capillary was incubated with the conjugated Fc-fragment (rh IgG1-Fc; R&D Systems) to serve as the negative control. Capillaries and hoses were rinsed with cell culture medium containing 10% FBS to block nonspecific binding before to be connected to the cell suspensions. Physiological shear stress [19] was produced with a syringe pump initially set to 0.5 dyn/cm for 3 min and subsequently raised to 1.0 and 1.5 dyn/cm for 3 min each. Rolling or adhesive cells were recorded and analyzed using a specially designed analyzing program (CapImage 8.5, Dr. Heinrich Zeintl, Heidelberg, Germany). Two independent experiments were conducted and analyzed.

2.5. Invasion assay

Cell invasion experiments were performed using the Matrigel Invasion Chambers constituted by 24 well plates equipped with 8 μm pore size polycarbonate filters overcoated with Matrigel (Corning Inc., New York, USA). LAN-1 (1×10^5) or SK-N-SH (4×10^4) cells were seeded in RPMI with 2% FBS in the upper compartment of each chamber. Medium with 10% serum was added to the lower compartment. BJ (ranging from 0.25% to 5%) was added to the upper compartment and after 24 h the cells which invaded to the lower side of the Matrigel coated filter were collected and counted in Neubauer hemocytometric chamber [20]. The test was repeated three times.

2.6. Wound healing assay

LAN-1 or SK-N-SH cells were cultured in 6 well plates in normal culture conditions until confluence. Thereafter, monolayer of NB cells was scratched using a thin sterile pipette tip, and culture dishes were washed with RPMI. Then, cultures were treated or not with BJ 1% for 12 h and the wounds were photographed by an inverted contrast phase microscopy. This assay has been remade three times.

2.7. Study in experimental tumors

Forty-eight male and female severe combined immunodeficient (SCID) mice, aged 9 ± 14 weeks (Charles River Laboratories, Sulzfeld, Germany), weighing on average 25 g (SEM ± 0.5 g) at the beginning of the experiments, were used. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care with stating the guidelines (Directive 2010/63/EU). The animal tests were approved by the local animal experiment approval committee (Behörde für Soziales, Familie, Gesundheit, Verbraucherschutz; Amt für Gesundheit und Verbraucherschutz, Hamburg, Germany) and assigned the project number 19/10.

SK-N-SH and LAN-1 NB cell inoculations were performed as described [21]. Cells were harvested by trypsination and suspended in cold culture media diluted 1:1 with Matrigel (Becton Dickinson GmbH, Heidelberg, Germany). Forty-eight mice were divided into two groups and inoculated with SK-N-SH and LAN-1 cells, respectively (24 mice per cell line). An aliquot of 200 μl (1×10^6 cells) of this suspension was injected subcutaneously between the scapulae of each SCID mouse.

One day after tumor cell inoculations, 200 μl of microfiltered *C. bergamia* juice was orally administered to the mice of the BJ-treated groups, while the mice of the control groups received 200 μl of sterilized water. Treatment was repeated once a day for a total of 28 days, at the same time. Common behavior (eating, drinking, climbing, social interactions) and signs of pain and distress (weight loss, ruffled fur, ocular discharge, lethargy, ataxia, labored respiration, hypothermia) were regularly recorded, and no signs of impairment caused by the treatment were noticed. Twenty-nine days after cell inoculation mice were terminally anesthetized with intraperitoneal injections of 0.1 ml/10 g body weight Rompun/Ketanest (0.8 ml Rompun 2% by Bayer, Leverkusen, Germany plus 1.2 ml Ketamin Gräub 100 mg/ml from Albrecht, Aulendorf, Germany in 8 ml 0.9% NaCl). The blood samples were transferred in EDTA microtubes and stored at 4 °C until use for DNA extraction. The primary tumors and lungs were removed and fixed in 4% paraformaldehyde (PFA) in PBS.

2.8. Lung metastasis detection

The lungs were fixed en bloc and cut into 1 mm thick slices, spread randomly over a glass slide and then embedded in 2% agar (Agar Noble; Difco Laboratories, Detroit, MI, USA). The solidified agar blocks were subsequently processed for routine wax histology and serially sectioned. Every tenth section was retained and ten sections from the middle of the block were stained with hematoxylin–eosin. The lung metastases were counted using a Zeiss Axioplan photomicroscope (Jena,

Germany). The total number of lung metastases was determined as described previously [22].

2.9. Analysis of tumor cells in the blood

In order to detect human tumor cells in the bloodstream, quantification of human tumor cell DNA *via* Alu sequences was carried out [23]. In brief, quantitative real-time polymerase chain reaction (qRT-PCR) and melting curve analyses were performed in the well of 96-well plate with the LightCycler® 480 1.5 System. For the qRT-PCR the LightCycler Fast Start DNA MasterPLUS SYBR Green I Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used. DNA was extracted from 200 μl of the blood by QIAmp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). Two microliter DNA was used as templates for the PCR reaction and incubated in a total reaction volume of 10 μl, containing 1× SYBR Green I Master mix including Taq DNA polymerase, Taq PCR buffer, a dNTP mixture, 1 mmol/l $MgCl_2$, and 10 pmol specific Alu primers. Forward Alu primer (TGG CTC ACG CCT GTA ATC CCA) and reverse Alu primer (GCC ACT ACG CCC GGC TAA TTT) were synthesized by MWG-Biotech AG (Ebersberg, Germany). The PCR conditions were initially 10 min 95 °C, followed by 50 cycles of 5 s 95 °C, 5 s 67 °C and 20 s 72 °C (measurement of fluorescence). Melting curve analysis (1 s 95 °C, 1 s 65 °C and 0 s 95 °C) was performed directly after PCR run. Quantification of disseminated tumor cells in the mouse blood was based on a standard curve using the blood spiked with *in vitro* grown SK-N-SH and LAN-1 NB cells (1 cell/ml up to 1×10^6 cells/ml).

2.10. Statistical analysis

MTT data were statistically analyzed by one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison test (GraphPad Software for Science, San Diego, USA). *Post-mortem* primary tumor weights of control and BJ-treated groups were compared by two-tailed Student's *t*-test. Numbers of pulmonary metastases and disseminated tumor cells in the blood, respectively, were logarithmized. Two-tailed Student's *t*-test was used to compare the values of control and treated groups. Logarithmized numbers of lung metastases were also evaluated by an analysis of covariance (ANCOVA) with factor logarithmized numbers of tumor cells in the blood and including interaction terms of mouse group * logarithmized numbers of tumor cells in the blood. This non-significant interaction was omitted (backward selection) and the effect estimated for the final model was presented with *P* value. These statistical tests were carried out using IBM SPSS statistics software (SPSS version 18.0 for Windows, IBM, Ehningen, Germany). $P < 0.05$ was considered as a statistically significant result. All data were visualized using GraphPad Prism 5.0.

3. Results

3.1. Flavonoid composition of bergamot juice

In this paper, the identification and quantification of the flavonoid content in BJ were attained by means of RP-HPLC in combination with UV and MS detectors. Identification of the bioactive molecules was carried out by LCMS detection and comparison with standard materials, when available. For all molecule classes studied in this work, the validation process provided good results for LOD and LOQ, ranging from 0.034 to 0.080 mg/l and from 0.047 to 0.113 mg/l, respectively. R^2 coefficients, ranging from 0.991 to 1.000 were attained for all the analytes, showing a good linearity for the method used. Fig. 1 shows the RP-HPLC/UV chromatogram of flavonoids in BJ. The flavonoid quantitative composition (mg/l ± standard deviation) in the BJ analyzed is reported in Table 1.

3.2. Effects of bergamot juice on neuroblastoma cell proliferation in vitro

Incubation of LAN-1 and SK-N-SH cells with growing concentrations of BJ ranging from 0.5% to 10% for 24, 48 and 72 h reduced cell proliferation in a concentration-dependent manner (Fig. 2). Concentrations of BJ up to 5% did not significantly influence cell growth of LAN-1, while SK-N-SH cells appear to be more sensitive to treatment, since BJ was effective also at concentrations of 1% (incubation time 24 and 48 h $P < 0.05$ *vs* control; incubation time 72 h $P < 0.01$ *vs* control) and 2.5% ($P < 0.001$). Moreover, 5% BJ incubation for 48 and 72 h decreased SK-N-SH cell proliferation of 80 and 90%, respectively, whereas in LAN-1 cells the growth inhibition reached about 50%. The strongest antiproliferative effect was obtained incubating the cells with 10% BJ for 72 h: a cell growth reduction of 95% in SK-N-SH and of 85% in LAN-1 is achieved under this experimental condition ($P < 0.001$ *vs* untreated cultures; Fig. 2A). MTT data were confirmed by those of the cell count assay (Fig. 2B).

In order to assess the cytotoxic effects eventually induced by the BJ either SK-N-SH or LAN-1 cells were incubated with different concentrations of BJ (from 1 to 10%) for 24 h, and then the trypan blue dye exclusion test was performed to detect dead cells. Fig. 3 shows that BJ induced significant increase in cell death in both cell lines only at the higher concentration (10%; $P < 0.05$ *vs* control), suggesting to pursue the *in vitro* studies with concentrations of BJ not greater than 5%.

3.3. The bergamot juice affects cell adhesion, invasion and migration in vitro

SK-N-SH cells showed adhesion on rh E-selectin–Fc-chimera coated surface (5 μg/ml). As shown in Fig. 4, treatment with 5% BJ reduced adhesive SK-N-SH cells per time unit and field of view (0.8 × 0.6 mm), especially under low shear stress of

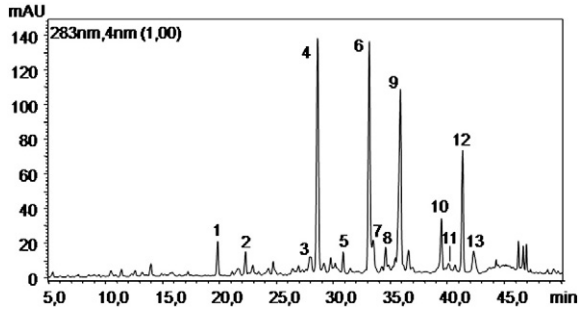

Fig. 1. RP-HPLC/PDA/MS chromatogram of flavonoids in bergamot juice. For peak identification see Table 1.

Table 1
Values of λ_{max} (nm), $[M-H]^-$, concentration (mg/l ± SD) and CV% of flavonoids in bergamot juice analyzed.

N°	Compounds	λ_{max}	$[M-H]^-$	mg/l	CV%
1	Apigenin-6,8-di-*C*-glucoside	272, 336	593	33 ± 1.8	5.52
2	Diosmetin-6,8-di-*C*-glucoside	272, 348	623	29 ± 1.9	6.63
3	Eriocitrin	252, 259	595 (287)[a]	24 ± 0.8	3.33
4	Neoeriocitrin	285	595	78 ± 3.5	4.46
5	Poncirin	224, 285	594	21 ± 0.9	4.28
6	Naringin	227, 284	579	111 ± 5.7	5.13
7	Narirutin	224, 285	579 (271)[a]	17 ± 0.3	1.76
8	Neodiosmin	253, 268, 349	607	15 ± 0.4	2.66
9	Neohesperidin	218, 285	609	84 ± 2.4	2.84
10	Naringenin	224, 285	723	34 ± 1.1	3.10
11	Rhoifolin	262, 339	577	9 ± 0.6	6.41
12	Hesperetin	225, 284	753	108 ± 3.9	3.60
13	Diosmin	224, 285, 342	607 (299)[a]	38 ± 0.5	1.32
	All			610 ± 21.5	3.52

[a] Between parenthesis the daughter ion value for compounds 3, 5, 10, 11, and 12 is reported.

0.5 dyn/cm^2 (control: 18.5 *vs* BJ: 4.5). LAN-1 cells rarely adhered to E-selectin independent of treatment with BJ, so that the effect of BJ on LAN-1 cell adhesion to E-selectin could not be evaluated. No adhesive events could be observed in the control capillaries incubated with the Fc-fragment and in the capillaries coated with rh P-selectin–Fc-chimera (50 μg/ml) fusion protein (data not shown).

In order to evaluate the metastatic potential of NB cells in the presence of BJ, further studies were focused on the effect of the juice on the SK-N-SH and LAN-1 cell invasiveness. As shown in Fig. 5, treatment with 0.5, 1 and 5% BJ significantly reduced the mean number of NB cells able to cross the Matrigel-coated filter within 24 h. 1% BJ decreased SK-N-SH and LAN-1 cell migration up to 50 and 60%, respectively, and 5% BJ produced decrement of about 70% compared with untreated cultures in both cell lines ($P < 0.001$). The anti-invasive effects of BJ were observed even at a concentration that did not induce any anti-proliferative or cytotoxic effects (0.5% BJ; −30% in SK-N-SH and LAN-1 cells; $P < 0.01$). The migratory capacity of NB cells in the presence of BJ was evaluated by the wound healing assay. Pictures of representative wounds after 12 h of incubation with or without 1% BJ are shown in Fig. 6, in which the impairment of the cell motility caused by the juice is appreciable (the wounds appeared to be of the same size as that of time zero). BJ concentrations higher than 1% increase weak adherent cells, not allowing a proper analysis of the cell motility.

3.4. Influence of bergamot juice on primary tumors and metastasis

Upon subcutaneous injection of SK-N-SH or LAN-1 cells development of primary tumors was found in 100% of SCID mice independent of treatment with BJ. Primary tumor weight varied considerably within the groups and was not significantly influenced by BJ. However, especially in LAN-1, the number of disseminated tumor cells in the blood was higher in BJ-treated mice than in control mice, although this difference was not statistically significant ($P = 0.057$; Fig. 7).

All control mice with LAN-1 primary tumors produced pulmonary metastases, with numbers ranging from 5461 to 284785; in the BJ treated group, all mice developed lung metastases with numbers ranging from 558 to 405058 (Figs. 8 and 9). The number of disseminated LAN-1 tumor cells in the blood showed a strong positive correlation with LAN-1 pulmonary metastases ($P < 0.001$). In the *t*-test analysis the number of lung metastases in BJ-treated LAN-1 SCID mice exhibited no difference with the control group. However, including the number of disseminated tumor cells in the blood as covariate LAN-1 lung metastases were significantly reduced by BJ treatment ($P < 0.01$) (Fig. 8C). Nine of ten control mice with SK-N-SH primary tumors produced pulmonary metastases (mean: 507); in the BJ-treated group eleven of twelve mice developed lung metastases (mean: 356). Though the mean of lung metastases in BJ treated SK-N-SH mice is lower than that in control mice, *t*-test analysis did not show a statistically significant difference.

4. Discussion

Metastasis formation is the spread of tumor cells from a primary tumor to distant sites in the patient's body. This process is the end result of a complex series of events depending on the ability of the tumor cells to detach from the primary tumor, to migrate and invade connective tissues, to enter into the vascular or lymphatic system and to achieve vital organs where tumor cells have to proliferate to form a distant metastasis. The tendency of a primary tumor to form metastases is the hallmark of malignant neoplasms and has important diagnostic, prognostic, and therapeutic implications.

NB is a devastating tumor, already metastatic in 70% of the patients at the time of diagnosis. NB metastases commonly affect distant sites including the lungs [24]. Moreover, several patients develop metastatic disease also after surgical removal of the primary tumor. Thus, the formation of distant metastases is still a major problem in the treatment of NB patients, and finding novel therapeutic strategies is of great interest in NB research because of the poor prognosis of children with disseminated stages. Inhibition of the metastatic potential of NB cells might prevent disease progression, even for patients with multiple tumors at diagnosis.

In the present study we demonstrate that BJ significantly affects SK-N-SH and LAN-1 cell proliferation *in vitro*, but failed to reduce primary tumor weight in an *in vivo* NB xenograft model. Moreover, we show anti-adhesive and anti-invasive properties of BJ *in vitro* and slight anti-metastatic activity in a metastatic LAN-1 NB xenograft model *in vivo*. In particular, BJ decreases LAN-1 lung metastases under consideration of disseminated tumor cells in the blood. Therefore, through the impairment of tumor cell adhesive properties BJ may contribute in inhibiting cancer cell progression.

The influence of BJ in metastasis formation was investigated in a SCID mouse xenograft model in which lacking functional B- and T-cells enables the investigation of human cancer cell metastasis in an entire organism. SK-N-SH and LAN-1 cells were chosen because of their high tumor take-rate and a great spontaneous metastatic potential after an acceptable time period of growth from subcutaneous injection of the tumor cells [21]. SK-N-SH and LAN-1 cells were subcutaneously injected between the scapulae of SCID mice and circulatory

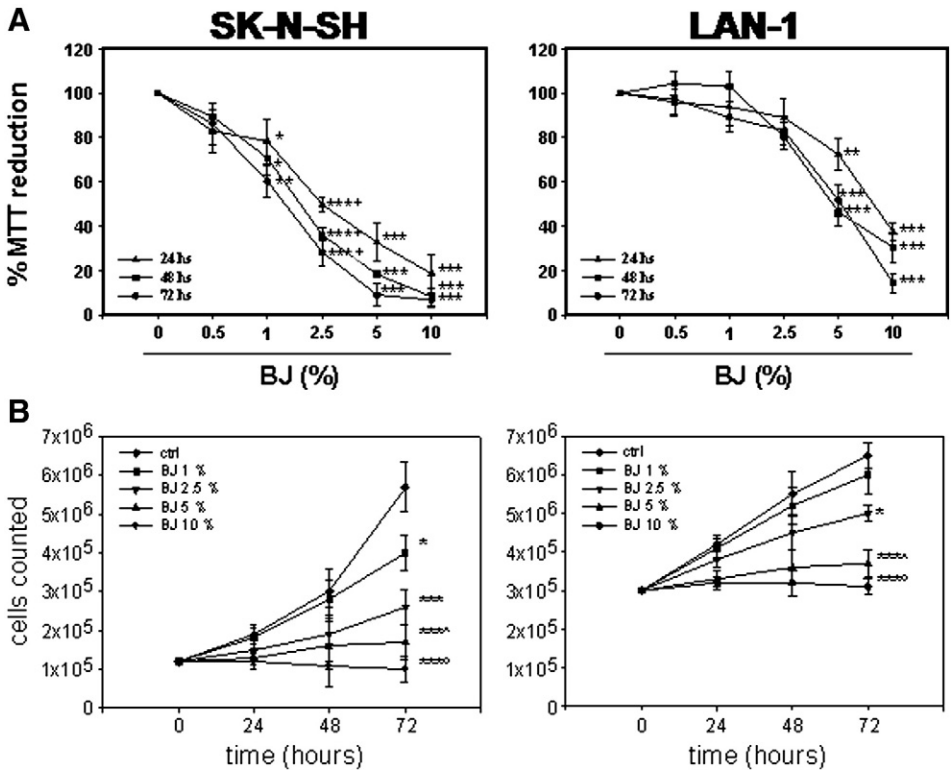

Fig. 2. Effect of BJ on SK-N-SH and LAN-1 cell proliferation. Cells were cultured in the presence of increased concentrations of BJ for 24, 48 and 72 h. Proliferation rates of SK-N-SH and LAN-1 cells were evaluated by MTT (A) and cell count (B) assays. Results of MTT are expressed as percentage of the values detected in untreated cells, while data of cell count are presented as the number of live cells counted. Each value is the mean ± SEM of three experiments performed in eightplicate (MTT test) or in triplicate (cell count). $^{*}P < 0.05$ *vs* ctrl; $^{**}P < 0.01$ *vs* ctrl; $^{***}P < 0.001$ *vs* ctrl and BJ 0.5%; $^{+}P < 0.05$ *vs* BJ 1%; $^{\wedge}P < 0.01$ *vs* BJ 1%; $^{\circ}P < 0.001$ *vs* BJ 1% (ANOVA, followed by Turkey–Kramer multiple comparison test).

dissemination is required to develop distant metastases. As already described by Nehmann and coworkers [23] we detected disseminated human tumor cells in small volumes of the mouse blood with the qRT-PCR for amplifying the repetitive character of human Alu DNA-sequences. BJ treatment increases the number of human NB cells in the bloodstream in both cell lines, but this result does not reach statistical significance. This finding indicates that BJ does not affect the detachment of NB cells from the primary tumor and the invasion in a blood vessel, but rather colonization of a distant site. Of interest, growing deal of experimental data suggests that progression to intractable fatal metastatic NB depends not so much on the ability of NB cells to dissociate from the primary tumor, but on the potential of these cells to invade distant sites and progress there [25]. Therefore, our findings appeared highly relevant in the light of several novel

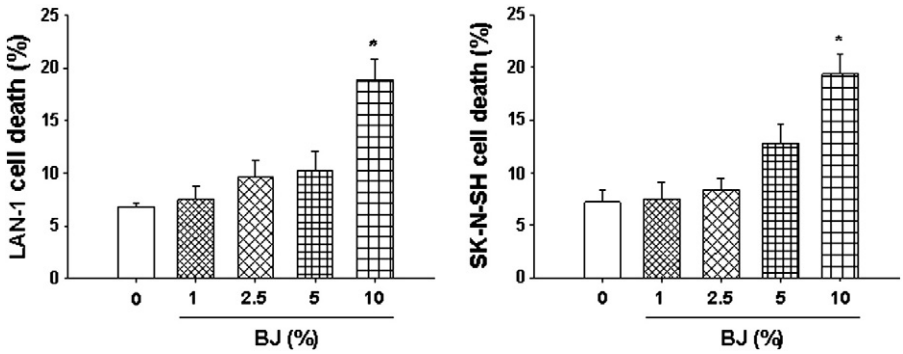

Fig. 3. Cytotoxic effects induced by BJ. BJ induces significant cytotoxic effects on SK-N-SH (on the right) and LAN-1 (on the left) cells only at the higher concentration used in this study (10%). Lower concentrations of BJ did not significantly affect cell viability. Cytotoxicity was evaluated in terms of cell death assessed by the trypan blue test. Data represent mean ± SEM obtained in the three different sets of experiments made in triplicate. $^{*}P < 0.05$ *vs* ctrl (ANOVA, followed by Turkey–Kramer multiple comparison test).

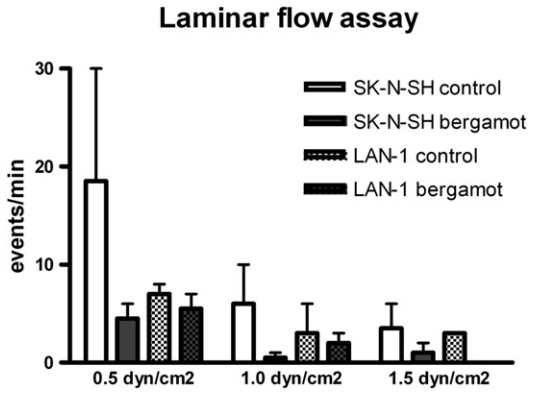

Fig. 4. Results of the laminar flow assay. Adhesion of untreated and 5% BJ-treated LAN-1 and SK-N-SH cells on rh E-selectin–Fc-chimera (5 μg/ml) coated microslides. The number of adhesive cells at different shear stresses (0.5, 1 and 1.5 dyn/cm^{-2}) per time unit and field of view. Data represent mean ± SEM of the two experiments.

anticancer therapies that do not necessarily lead to a tumor volume or weight reduction [26].

Considering disseminated tumor cells in the blood as covariate, BJ reduced the number of pulmonary metastases in LAN-1. Earlier *in vivo* findings in a colon cancer xenograft model showed that the knock-out of E- and P-selectins leads to a decrease of pulmonary metastasis and an increase of circulating tumor cells as compared to the wild type SCID mouse [18]. This increase of tumor cells in the bloodstream is most likely due to the lack of attachment of the tumor cells to the selectin deficient endothelium at the site of the future metastasis and the subsequent transmigration of the tumor cells through the endothelium. However, LAN-1 cells hardly adhere to selectins *in vitro* (Fig. 3) indicating that extravasation of LAN-1 cells is independent of selectin expression on endothelial cells. Probably, these cells make use of other cell adhesion molecules with regard to extravasation and diapedesis, both are requirements for a successful metastasis. In this line, we recently showed that BJ inhibited SH-SY5Y neuroblastoma cell adhesion on endothelial cell monolayer or other physiologic substrates [13]. Reduction of both cell adhesiveness and cell motility involves an impairment of actin filaments, a reduction in the expression of the active form of focal adhesion kinase (FAK) and neural cell adhesion molecule (NCAM), hindering the association between these two important molecules.

Over the past twenty years, numerous studies have shown the beneficial properties of flavonoids leading to increased attention to the *Citrus* juices which are an important source of these biomolecules. Their flavonoidic composition may considerably differ with regard to numerous factors such as the species, subclass, environmental conditions, crop methods and harvest time. However, the juice of hand squeezed *Citrus sinensis* is characterized by the presence of Hesperidin, Narirutin and Vicenin-2. Generally, commercial orange juices present a composition similar to freshly squeezed ones, with some compounds such as Naringin and Diosmin that indicate the possibility of a non-pure orange juice. *Citrus limon* juice is characterized by the presence of significant amounts of the flavanones Hesperidin and Eriocitrin. Commercial lemon juices reflect the composition of the hand-squeezed ones, with the exception of Hesperidin that often decreases. Among the many *Citrus* species the *Citrus aurantifolia* is the one that is most commonly referred to as "limes" and its composition closely resembles the flavonoid content of lemon juice. *Citrus paradisi* (grapefruit) juice can generally be found in three different varieties, red, pink and white, whose color depends on the presence (or absence) of lycopene. Generally, white grapefruit juice is slightly richer in flavonoids than the pink and red varieties. The main components are the flavanones Naringin and Naringenin, its aglycone, which has always been recognized to be a distinctive component of grapefruit juices. Narirutin is also present in good amounts. *C. bergamia* hand-squeezed juice is characterized by the presence of good amounts of the flavanones Poncirin, Naringin, Neohesperidin and Neoeriocitrin. Diosmetin 6,8-di-C-glucoside and Apigenin 6,8-di-C-glucoside are the most abundant flavones, hinting that *C. bergamia* may indeed be derived from a hybrid of *C. limon* and *C. sinensis*. Industrial BJ has been found to be extremely rich in flavonoids among which Neoeriocitrin, Naringin and Neohesperidin are the most abundant flavanones, whereas Apigenin di-C-glucoside, and Diosmetin di-C-glucoside are the greater plentiful glycosyl flavones. Commercial juices tend to be even more concentrated than their hand-squeezed equivalents, resulting in higher concentrations of the main components [27]. This very different flavonoidic composition of the various *Citrus* juices is reflected in their heterogeneous biological activity.

Several *in vivo* studies suggested the potential of *Citrus* juices in preventing cancer [7–11], but to our best knowledge, the scientific literature does not provide evidences on their anti-metastatic activity. This is the first report suggesting the

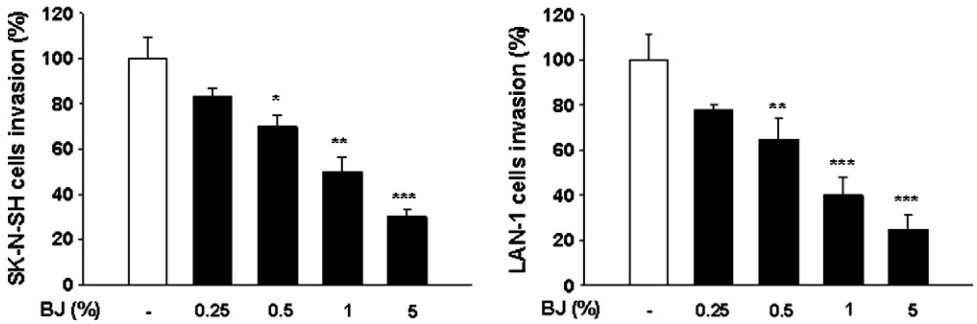

Fig. 5. Reduction of NB cell invasiveness by BJ. LAN-1 and SK-N-SH cells treated (filled bar) or not (empty bar) with BJ 0.25, 0.5, 1 and 5% migrating through the filter of Matrigel invasion chambers. Data are reported as percentages of cells counted in untreated cultures. Each value represents the mean ± SEM of the three sets of experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *vs* respective controls.

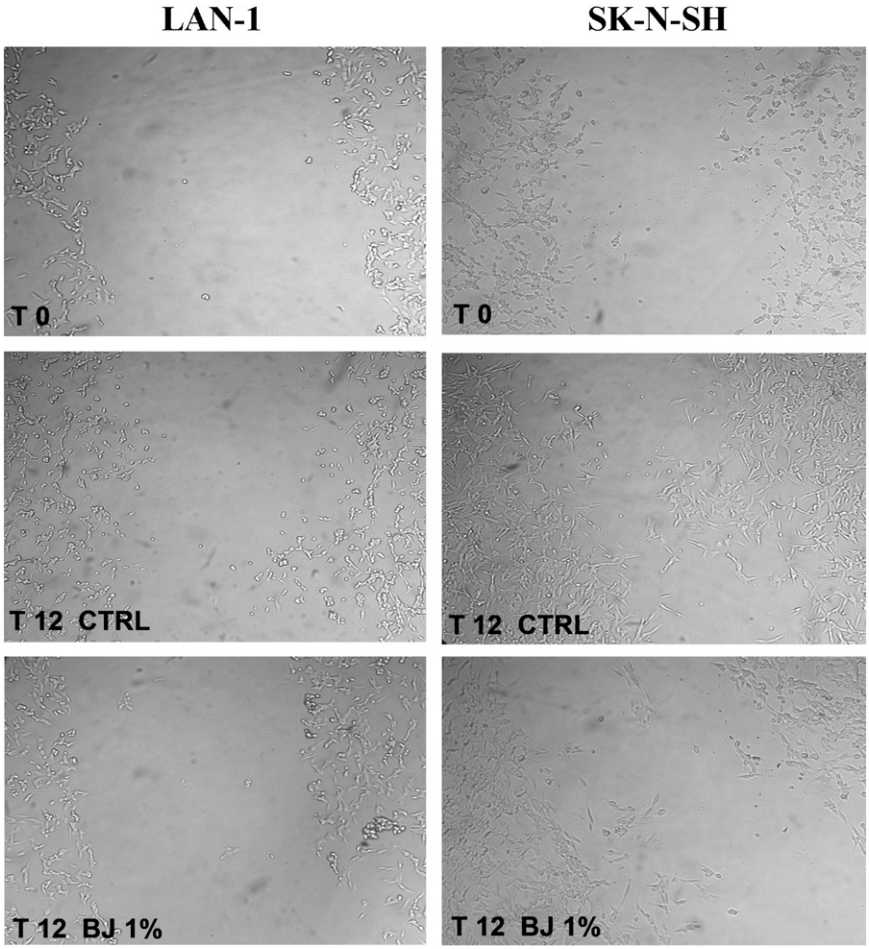

Fig. 6. BJ impairs NB motility. Several wounds were traced in the NB cell monolayer and a photograph was taken (T 0). Then, complete medium with or without BJ 1% was added and the migration of cells in the wound was photographed after 12 h. Representative images were taken.

potential of a *Citrus* juice in reducing metastasis colonization. Growing deal of data suggests that *Citrus* flavonoids may have inhibitory effects on cancer invasion and migration, indicating their capability to prevent cancer metastasis. Nobiletin seems to be the most promising anti-metastatic molecule because of its ability to suppress intracellular pathways involved in the metastatic process [28–30]. Apigenin, Quercetin, Naringenin and Hesperidin have also shown to reduce invasion and metastasis of tumor cells through modulation of key molecules implicated in the processes of metastasis formation [31]. The peculiarity of our study is that we used a natural bergamot juice and not juice with commercial bioactive molecules, which strengthens the importance to study the potential anticancer activity of fruits and vegetables by using

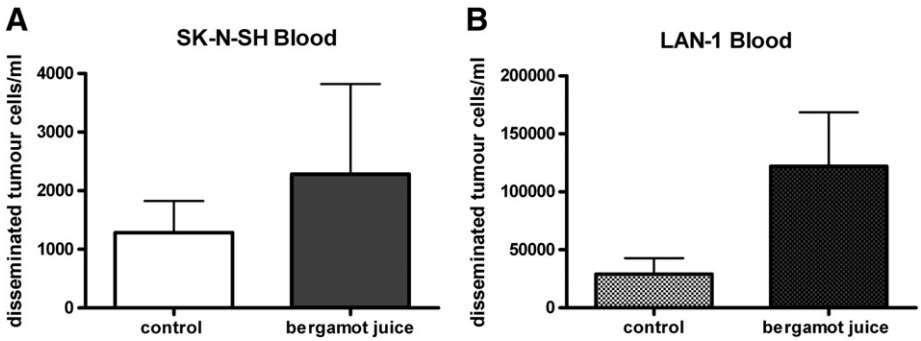

Fig. 7. Quantification of human tumor cell DNA in the blood of SCID mice. Disseminated tumor cells (DTCs) in blood (cells/ml) of SK-N-SH- (A) and LAN-1-SCID mice (B) were detected by quantification of human Alu DNA-concentrations using qRT-PCR. The mean expression levels of all measurements ± SEM are shown.

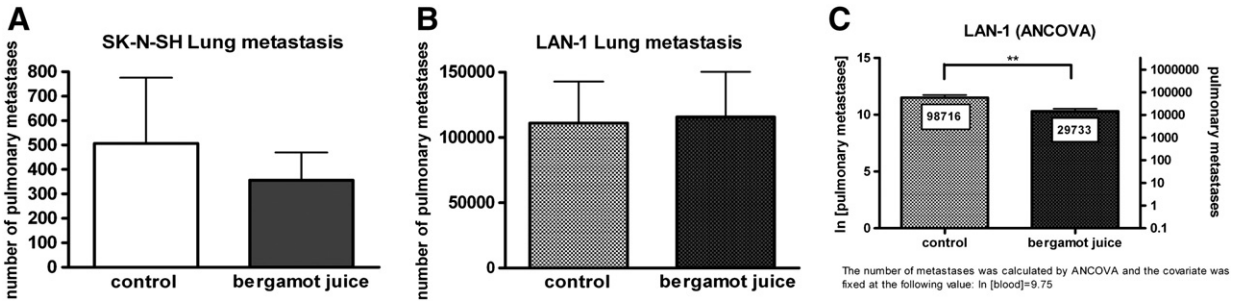

Fig. 8. Number of spontaneous lung metastases grown in SCID mice. Data, expressed as mean ± SEM, indicate values obtained in BJ-treated and untreated SK-N-SH (A) and LAN-1 SCID mice (B) group. Considering tumor cells in the blood as covariate, treatment with BJ significantly reduced the number of pulmonary metastases (C).

whole extracts containing all phytochemicals. Indeed, according to other researchers, we believe that the complex mixtures of phytochemicals present in fruits and vegetables could be more effective than their individual constituents in preventing cancer through both additive and synergistic effects [32]. This hypothesis is also supported by several case–control studies documenting the inverse association between *Citrus* fruit intake and risk of several types of cancer, including tumor of the digestive and respiratory tracts, pancreas, prostate, breast, nasopharynx and cutaneous melanoma [33–37].

In conclusion, this study demonstrates that BJ exerts significant inhibitory effects on human NB cell proliferation *in vitro* and suggests that the slight inhibitory effects on lung metastasis colonization *in vivo* may be due to the impairment of NB cell adhesiveness, migration and invasion observed *in vitro*. A lack of any apparent sign of systemic toxicities suggests a promising role of BJ in oncologic field, demanding further investigation in cancer and other chronic diseases. Finally, our findings appear very relevant because the results shown in this study were obtained using a natural BJ, which may be converted in a commercial product and used as a supplement in the healthy field.

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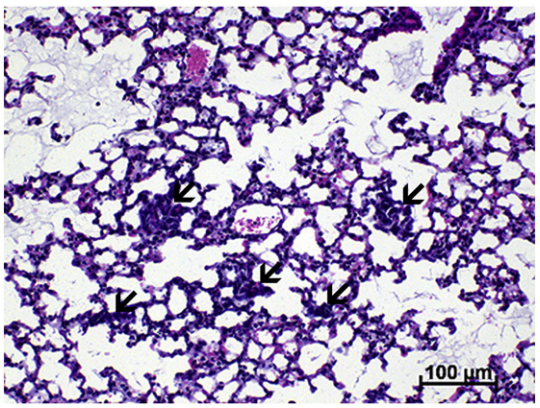

Fig. 9. Lung metastases of human LAN-1 cells engrafted in SCID mice. Arrows mark the pulmonary metastases produced by LAN-1 in mice treated with water.

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